

• AFLATOXICOSIS: THE PRESENCE OF AFLATORINS OR THEIR
METABOLITES IN LIVERS AND SKELETAL MUSCLE OF CHICKENS

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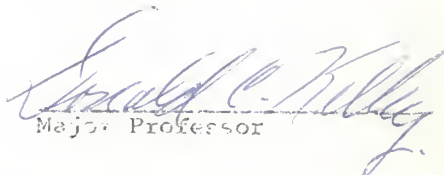
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INTRODUCTION

Since the discovery of aflatoxins early in this decade (Sargeant, et al., 1961b) interest in this group of toxins produced by certain strains of a mold, Aspergillus flavus, has provided a long needed impetus to mycotoxicological investigations.

Although numerous papers have been written on the biological, chemical and toxic properties of the toxin, Hesselstine, et al. (1966) stated, "The question regarding the fate of aflatoxin ingested by farm animals in milk, meats and eggs has been inadequately explored ... The fate of each of the aflatoxins needs to be studied in animals and their tissues, particularly those destined for human consumption."

A toxic substance has been reported in milk of animals receiving aflatoxins (Allcroft and Carnaghan, 1963; de Jongh et al., 1964b; vander Linde et al., 1965; Purchase, 1966; Mabney et al., 1967). To date, no definite evidence has been presented to substantiate or negate the possibility of aflatoxins in other foods of animal origin.

Since the toxic effects of aflatoxins in humans is unknown (Banes, 1966), demonstration of the toxins (which are among the most hepatotoxic and carcinogenic known) in such foods could be of public health significance.

This study was undertaken to determine the presence of the toxins or their metabolites in livers and musculature of chicken. Birds could be ingesting aflatoxins and conceivably used for human consumption.

In this study chickens were given crude aflatoxins in varying amounts daily for 6 weeks with the intent of producing mild to very severe toxic effects. Extraction and chromatographic procedures were adapted to attempt recovery of the toxins or their metabolites in avian tissues.

REVIEW OF LITERATURE

THE DISCOVERY OF AFLATOXIN. In 1960, outbreaks of what was first designated as "Turkey X" disease was reported in England by Blount (Spensely, 1963). In a period of just a few months approximately 100,000 turkey poultts died of the condition. Also affected were ducklings, pigs and calves. The common denominator in all of these outbreaks was a peanut meal imported from Brazil (Asplin and Carnaghan, 1961; Loosemore and Markson, 1961; Loosemore and Harding, 1961).

The disease in calves was first attributed to seneciosis because of the similarity in symptoms and liver lesions, but Allcroft et al. (1961), by chemical examination of the suspect feed, showed that no pyrrolizidine alkaloids or their N-oxides were present.

It was learned from further investigations that methanol and chloroform extracts of the peanut meal (including those of some Indian and African peanut meals) were toxic to ducklings. This knowledge provided the basis for the first bioassays of the toxin (Sargeant et al., 1961a).

Sargeant et al. (1961b) identified the toxic substances as metabolites of a mold which was identified by J. J. Elphick of the Commonwealth Mycological Institute to be Aspergillus flavus, Link.

In 1962, an interdepartmental working party in England gave the collective name of aflatoxin to these metabolites (Carnaghan et al., 1966).

GENERAL CHARACTERISTICS OF AFLATOXIN. Aflatoxin is composed of at least 4 difurano coumarin derivatives which when separated by thin layer chromatography (TLC) are observed to fluoresce under ultraviolet light in descending order as blue (B₁ and B₂) and green (G₁ and G₂) spots (de Jongh et al., 1962; Hartley et al., 1963; Asao et al., 1963, 1965). Excitation of fluorescence is maximal at a wavelength of 425 to 450 mμ. (Pons and Goldblatt, 1965).

Aflatoxins are relatively stable compounds. They can survive autoclaving (Hesseltine et al., 1966; Wogan, 1966) and are resistant to a number of chemical agents (Trager and Stoleoff, 1967). They are sensitive, however, to oxidation, light and some bacterial actions (Ciegler et al., 1966).

Of the four aflatoxins, B₁ is the most toxic. Relative toxicity may be best illustrated by the following single dose LD₅₀ in day-old ducklings: B₁, 18.2 ug; B₂, 84.3 ug.; G₁, 39.2 ug.; and G₂, 172.5 ug. (Carnaghan et al., 1963).

Naturally occurring aflatoxin has been found in relatively few commodities and has usually been associated with Aspergillus flavus. In addition to peanut meal, as mentioned above, cotton seed meal cake was found by Loosenore et al. (1964) to be contaminated with as much as .5 ppm aflatoxin as determined by duckling bioassay.

Other species of molds reported to be aflatoxin producers include Penicillium purpurescens (Hodges et al., 1964) and A. parasiticus which

is apparently one of the most active aflatoxin producers in the A. flavus-oryzae group (Codner et al., 1963.)

GENERAL CHARACTERISTICS OF ASPERGILLUS FLAVUS. Optimum temperature for growth of A. flavus is approximately 30 C, but it will grow in a temperature range of 12 to 47 C. It requires a relative humidity of 80 to 85% and a food moisture content of at least 18% (Raymond, 1966). Although crops may become contaminated with it in the field, most workers agree that it is primarily a storage mold and crops, especially peanuts, if insufficiently dried and improperly stored present the greatest aflatoxin hazard. Laboratory studies have shown that this mold can grow on any agricultural commodity and because of its worldwide presence it is likely that cases of aflatoxicosis have gone undetected prior to 1960. By the same token, it is not unlikely that more reports will appear in the future.

Morphologically, A. flavus differs from other species of the genus in that the conidial heads are globose to columnar and light yellow-green to olive-brown; conidiophores are usually colorless and roughened; conidia are round and usually roughened; and vesicles are fertile over most of their surface. On Czapek's agar the growth is tight velvety to coarse in texture and yellow-green to jade or olive-green (Raper and Fennel, 1965).

Aspergillus flavus, in addition to aflatoxin, produces several other toxins, viz, oxalic acid, Kojic acid, a tremoric substance, aspergillic acid and related antibiotics, Beta - nitropropionic acid and A. flavus endotoxin (Wilson, 1966).

BIOLOGICAL EFFECTS OF AFLATOXIN.

Symptoms and Species Differences. The first cases of "Turkey X" disease showed birds dying within one week, during which they developed anorexia, listlessness and alar weakness. Ataxia, convulsions and opisthotonus developed just prior to death (Spensely, 1963).

Allcroft (1965) reviewed the experimental effects of aflatoxin poisoning in feeding trials with farm animals and discussed the gross and histopathologic findings. In these preliminary trials, susceptibility varied in different species with the young being more susceptible than older animals. Ducklings were particularly susceptible, turkey poults less so and chickens appeared comparatively resistant but showed a reduction in growth rate. In the larger farm animals, pigs from 3 to 12 weeks, pregnant sows and calves 1 to 6 months were very susceptible to rations containing highly toxic peanut meal. Older cattle were more resistant but milk production was decreased. Sheep were resistant.

Hintz (1967) reported that as little as 810 ppb aflatoxin in rations fed to growing finishing pigs greatly reduced weight gains. Newberne et al. (1966) demonstrated strong circumstantial evidence that the cases of hepatitis X in dogs which had received a commercial diet containing peanut meal (Siebold and Bailey, 1952; Newberne et al., 1955) paralleled those of aflatoxin B₁ poisoning. In the experimental disease, dogs died without showing any symptoms and no difference was observed between oral and intraperitoneal routes of administration. It was demonstrated that dogs were susceptible to lethal effects in the same dose range as ducks, guinea-pigs and rabbits. Tulipula et al.

(1964) demonstrated subacute effects in 1.5 to 2.0 Kg. rhesus monkeys. Five tenths to 1.0 mg/day for 18 days followed by 1.0 mg/day thereafter resulted in anorexia and death in 14 to 28 days.

In general it can be said that the degree of toxicity of aflatoxins in animals depends on the dose rate, length of exposure, age and specie of the animal. Brown and Abrams (1965) indicated that in chickens breed can also be a factor. Wogan (1966), in the most complete review of biological effects of aflatoxins to date, reported sex differences in the rat.

Macroscopic lesions. Liver damage is the primary lesion observed in all species studied. Macroscopically, there is pallor and mottling of the liver with some degree of visceral edema and ascites. Carnaghan et al. (1966) reported enlargement of the liver in chickens, together with pale color and petechial hemorrhages early in the disease, followed by increasing firmness and the appearance of nodular areas after the 7th week. Cardiner and Oldroyd (1965) reported pale areas resembling lesions of white muscle disease in the musculature of chickens in field cases of avian aflatoxicosis.

Microscopic lesions. Bile duct proliferation with periportal necrosis, is typically present in affected species. Fibrosis is a common feature of chronic poisoning in pigs and cattle but this is not so well defined in the avian liver (Allcroft, 1965). Clegg and Bryson (1962) and Hill (1963) reported a veno-occlusive phenomenon which apparently is confined to the bovine liver. Svoboda et al. (1966) stated that chronic liver changes in the monkey closely resembled those observed in human viral hepatitis.

In chickens, Asplin and Carnaghan (1961) reported that hepatic cell degeneration and bile duct proliferation occur during the first 2 to 3 weeks, then disappear and are followed by massive lymphoid cell infiltration. Microscopic changes progress from fatty change to ductule cell proliferation, hemorrhages, enlargement of the parenchymal cells with nuclear degeneration to bile duct proliferation, severe inflammatory reaction, regeneration of parenchymal cells and fibrosis (Carnaghan *et al.*, 1966). Histologically, the muscle lesions observed by Gardiner and Oldroyd (1965) were characterized by large areas of necrosis, a diffuse increase of sarcolemmal nuclei, fragmentation and loss of fibrillar structure with interstitial edema in chickens receiving toxic peanut meal for 21 days.

Brown and Abrams (1965) observed the mitochondria to be enlarged 2 to 3 times in livers of chickens with acute and chronic aflatoxicosis.

Biochemical changes in chickens such as RNA/DNA ratios, lipid content, vitamin A content, liver and serum enzymes and serum protein have also been reported by Brown and Abrams (1965) and Carnaghan *et al.* (1966).

Other biological effects. The teratogenicity of aflatoxin B₁ in hamsters was demonstrated by Elis and DePaolo (1967). They found that a single intraperitoneal injection of 4 mg./kg. in pregnant females resulted in malformations ranging in severity from umbilical hernias to anencephaly. Histologically there were severe toxic effects in livers of both mothers and offspring.

The carcinogenic effect of aflatoxin has been well established in several species. Barnes and Butler (1964) demonstrated that 2.5 mg.

aflatoxin fed to rats over a period of 89 days resulted in hepatic carcinomas up to one year later. In comparing this dose to those of several well known carcinogens, Butler (1965) indicated that aflatoxin is the most active hepatocarcinogen known. Newberne (1965) demonstrated the carcinogenicity of aflatoxin contaminated peanut meal, grown and processed in the United States, in rats, ducks, and mice. Halver (1965) reported aflatoxin induced hepatomas in rainbow trout which are more susceptible than rats. Svoboda et al. (1966) reported hepatocellular carcinomas in monkeys receiving chronic doses of B₁.

The mechanisms by which aflatoxin exerts its toxic effects are not altogether understood. In livers of affected chickens and ducklings, Brown and Abrams (1965) observed a marked decrease of certain mitochondrial dehydrogenases and enzymes of the electron transfer chains or oxidative phosphorylation mechanisms and suggested that the suppression of protein synthesis, especially the albumin fraction, is due to a lower rate of ATP synthesis consequent to mitochondrial injury. Zuckerman and Fulton (1966), in studies with human embryonic liver cell cultures, reported that concentrations of 10 ppm B₁ produced marked changes to complete loss of RNA to total cell death in 40 to 44 hours. Clifford and Reese (1967) detected aflatoxin B₁ in the nuclei of liver cells from affected rats and demonstrated in vitro that it interacted with DNA thereby inhibiting production of nuclear RNA. This interaction suggests an explanation for the inhibitory action of aflatoxin on mitosis and its necrogenic action. Legator (1966) states that aflatoxin affects biological systems in a manner similar to alkylating agents which are mutagenic, carcinogenic and

antineoplastic. The latter property as applied to aflatoxins has yet to be demonstrated.

INVESTIGATIONS OF AFLATOXIN AND ITS METABOLITES IN ANIMAL TISSUES AND FOOD OF ANIMAL ORIGIN. Allcroft and Carnaghan (1963), de Iongh et al. (1964b) and vander Linde et al. (1965) reported that milk from cows receiving aflatoxin contaminated feed contained a toxic principle which produced biological effects in ducklings similar to those produced by aflatoxin. This toxic principle was isolated by thin layer chromatography and appeared as a blue-violet spot at a lower R_f than standard aflatoxins. de Iongh et al. (1964b) postulated that this substance was probably a derivative of B_1 since lactating rats receiving only purified B_1 exhibited the same spot. Similar findings of the "milk toxin" were reported by Purchase (1966) in milk of goats and cows which received 10 and 12 mg. per day, respectively.

Brown and Abrams (1965) could not demonstrate any toxicity in milk of cows receiving highly toxic peanut meal and attributed their negative results to the apparent inherent resistance of their cattle to aflatoxin. Furthermore, they fed the flesh of ducklings suffering from biochemically and histopathologically confirmed aflatoxicosis to New Hampshire chickens (the most susceptible to aflatoxin of 5 breeds tested) without any observable differences between the control and test animals. They concluded that, from a practical point of view, skeletal tissue contains very little or no aflatoxin in affected animals. Platnow (1965) was unable to extract aflatoxins or their metabolites from skeletal muscle and livers of chickens fed toxic peanut meal for periods up to 6 weeks.

Allcroft and Carnaghan (1963), using ducklings for assay, demonstrated no toxicity in liver, clotted blood and serum from cows receiving toxic rations, a pig's liver (taken from an animal with fatal aflatoxicosis) or pullet eggs from hens on a highly toxic ration.

On the other hand, Butler and Clifford (1965) extracted livers, portal blood and systemic blood of rats which had received a single LD₅₀ dose (7 mg./kg.) of B₁ orally or intraperitoneally. On TLC they demonstrated B₁ and a spot corresponding to "milk toxin" in all 3 extracts one half hour after dosing. Both spots were present in liver extracts up to 24 hours after dosing. In preliminary experiments, Van Zytveld¹ extracted B₁ and G₁ from livers and skeletal tissue of 150 to 200 Gm. guinea pigs which had received crude aflatoxins representing a total of 180 ug. B₁ and 160 ug. G₁ over a 12 day period. Similarly, a slow running blue spot and greenish spot at a lower R_f than aflatoxins were present on TLC.

Although sheep are resistant to the toxicity of aflatoxins, Nabney et al. (1967) in excretion studies of a lactating ewe, reported the "milk toxin" was present in feces, urine and milk after the animals received a single dose of 1 mg./kg. total aflatoxins. Wogan (1966) in studies with C¹⁴ labeled aflatoxin B₁ injected intraperitoneally in rats, demonstrated that 25 to 30% of the C¹⁴ was recovered in CO₂, 25% was excreted in the urine, 25% in the feces and intestinal contents and 6 to 9% was recovered from the liver. The chemical nature of the excreted compounds has been determined by Holzapfel et al. (1966) and

¹ Unpublished observations.

although it is generally accepted that the metabolism of aflatoxin occurs primarily in the liver, the metabolic pathways are not fully understood.

LABORATORY PRODUCTION OF AFLATOXIN. Since relatively large quantities of aflatoxin are required for animal experimentation, investigators are usually obliged to produce it themselves. From a practical standpoint, it is convenient to grow the mold on a sterile feed type substrate which can be incorporated into a ration whose B₁ content can then be expressed in ppm or ppb². Crude and purified aflatoxins can be extracted from natural or synthetic media for more controlled experiments.

Hesseltine et al. (1966) reviewed a number of methods of aflatoxin production in which, for the most part, various cereal grains were used as the substrate. These workers also described a method by which yields totaling 1.4 mg./Gm., B₁ and G₁ on sterile wheat fortified with methionine could be achieved. This yield was much greater than any previously reported. Codner et al. (1963) had reported production on sterilized peanuts with yields of 265 mg./kg. Stubblefield et al. (1967) reported yields of 1.8 mg./Gm. total B₁ and G₁, on wheat solid substrate.

The metabolic chain of events in the synthesis of aflatoxin is not understood. In trials using semisynthetic media, Mateles and Ayde (1965) demonstrated the requirement of zinc and that casamino acids are the preferred nitrogen sources for aflatoxin production. Furthermore, they reported that sucrose, glucose and fructose can be used as sources of carbon but not lactose, mannose, xylose or sorbose.

²Dr. A. F. Keyl, Head, Pharmacology Section, Western Utilization Research and Development Div., USDA, Albany, Calif. Pers. com., March, 1967.

Using a basal medium of yeast extract and sucrose in distilled water, Davis et al. (1966a) reported yields of B₁ and G₁ up to 63 mg./100 ml. media, in a stationary culture. Hayes et al. (1966) reported lower yields with the same strain of mold in the same medium when cultured in aerated fermentors. Mateles and Ayde (1965) had similar results in that yields were greater in shaken cultures but less in aerated fermentors.

The method of production currently used by the Division of Microbiology, Food and Drug Administration is one developed by Hodges and Nesheim³. It consists of growing the toxic mold on sterile pulverized shredded wheat. The toxin is recovered in a crude powdered state by extraction with boiling chloroform and precipitation with petroleum ether.

Production of aflatoxin is usually maximal at 24 to 28 C and is independent of maximum mycelial growth (Davis et al., 1966a; Hesseltine et al., 1966; Schindler et al., 1967; Stubblefield et al., 1967). Maximum production is attained in 4 to 7 days. After that time extractable aflatoxins are decreased, probably due to some enzymatic action (Hesseltine et al., 1966) while mycelial growth increases.

It is also important to note that not all strains of A. flavus produce aflatoxin and with those that do the yield varies (Hesseltine et al., 1966; Codner et al., 1963).

EXTRACTION AND ASSAY PROCEDURES. Methods employed consist of extraction by a solvent such as methanol or chloroform, a cleanup process involving

³Dr. A. D. Campbell, Chief, Contaminants Branch, Div. of Food Chemistry, Bureau of Science, Dept. of H. E. W., FDA, Washington, D.C. 20204. Personal Communication, March, 1967.

paper or column chromatography and separation of the four fractions by thin layer chromatography.

Trager et al. (1964) compared 6 different methods employed by various government and industrial laboratories. In all methods compared alumina gel was used as the TLC adsorbent except the then current Association of Official Analytical Chemists (A.O.A.C.) method in which silica gel was specified. The silica gel was found to be superior to alumina gel. However, all methods except one were comparable when silica gel was used as the adsorbent. Methods at this time, especially the cleanup processes, were cumbersome, entailing a good deal of time and equipment. Quantitative assays were still unreliable. de Jongh et al. (1964a) developed a semi-quantitative assay procedure which employed extracts representing only 40 Gm. of sample. Improvement in quantitation and speed was reported by Nesheim et al. (1964), Campbell et al. (1964), Pons and Goldblatt (1965), Heuksinveld et al. (1965) and Wiley (1966).

The simplest, most rapid, yet reliable chemical assay developed thus far was described by Eppley (1966). Fifty Gm. and 1 kg. samples can be extracted by the methods described. Using a 50 Gm. sample, the results have been shown to compare excellently with the current A.O.A.C. official, first action method⁴. Because of the improved cleanup process, extracts equivalent to about 1 Gm. of sample can be spotted and chromatographed compared to the 0.2 Gm. sample with the A.O.A.C. procedure.

⁴"Changes in Methods", J.A.O.A.C., 49, (1), 1966, 229-231.

In all the above methods quantitative measurements are based on visual comparison of intensity of fluorescence in the sample spots to that of known aflatoxin standards on TLC plates.

Quantitation of aflatoxin in solution by spectrophotometry has been developed by Nabney and Nesbitt (1965). The use of a recording densitometer for quantitation of separated aflatoxins on TLC plates has been described by Stubblefield et al. (1967)

Bioassays for the toxicity of aflatoxins extracted from foodstuffs include the day-old duckling test, (vide supra) which is still currently accepted as a standard method by many laboratories. The chick embryo test (Verret et al., 1964) is one of the bioassays employed by the FDA. This test requires 21 days for completion but considerably less amounts of aflatoxin (LD_{50} is 0.025 ug. in the air cell) compared to the duckling assay. Another bioassay for toxicity of crude and purified aflatoxin B_1 consists of subjecting tissue cultures of diploid and heteroploid human embryonic lung cells to the toxin. Toxic effects in concentrations as low as 0.01 ug. aflatoxin can be observed in suppression of mitotic division which is maximal in 3 to 12 hours (Legator and Withrow, 1964).

A chemical confirmatory test of aflatoxin B_1 has also been adopted as official, first action by the FDA. This test, developed by Andrellos and Reid (1964), consists of reacting the sample aflatoxin with trifluoroacetic acid, formic acid/thionyl chloride and acetic acid/thionyl chloride. The chromatographically altered compounds are compared to those of standard aflatoxin B_1 reacted with the same three compounds on TLC.

MATERIALS AND METHODS

AFLOATOXIN PRODUCTION. Aflatoxin production was accomplished with slight modification of the method described by Davis et al. (1966a). A lyophilized culture of Aspergillus flavus, ATCC 15517⁵, was reconstituted in 0.3 ml. of YEM broth (3 Gm. each of yeast extract and malt extract, 5 Gm. peptone, 10 Gm. glucose and 1000 ml. distilled water autoclaved for 15 minutes at 15 psi). A 0.15 ml. portion was transferred to a foam stoppered tube containing approximately 10 ml. of the same broth and incubated for 3 days at 23 to 25 C. Loop inoculations from the YEM broth were made on slants of Czapek's solution agar⁶ modified with 7 Gm./l. of yeast extract and 30% sucrose.

These cultures were incubated at room temperature for 10 days to 2 weeks. To each of 10 such slants, 10 ml. sterile YES medium (2% yeast extract plus 20% sucrose in distilled water) was added and suspensions of the spores was made by gently agitating the growth with a sterile loop.

Spore suspensions were transferred to 10 foam stoppered Roux flasks containing 90 ml. of sterile YES medium. Loop transfers were also made to another 10 culture slants from the spore suspensions to be used for the next batch of aflatoxin production.

The Roux flasks were placed in an incubator at 25 C for 6 to 8 days when aflatoxin production was expected to be maximal.

⁵American Type Culture Collection, Rockville, Maryland.

⁶Difco Laboratories, Detroit, Michigan.

The mycelial mats were broken up with a glass rod and the entire contents of the flasks were filtered through fluted 50 cm. filter paper⁷ in an appropriately sized glass funnel. The filtrate was collected in a 2 liter Erlenmeyer flask, refiltered by filter paper⁸ in an 18 cm. Buchner funnel under negative pressure and finally collected in a 1 liter screw cap bottle. Asepsis was maintained throughout the entire procedure. All *A. flavus* cultures were handled almost exclusively in an enclosed hood.⁹

Ten such batches were produced and maintained at 4 C for later use. Batches 1, 2 and 3 were combined for use in the first experiment while batches 6, 7, 8, 9 and 10 were used for the second. Bottles containing the toxic medium were tightly wrapped in aluminum foil. Liquid YES medium was also prepared and stored similarly for use in control animals.

AFLATOXIN ASSAYS OF YES MEDIUM.

Extraction. One half ml. of medium was shaken for 1 hour with 100 ml. chloroform in a separatory funnel on a mechanical wrist action shaker.¹⁰ Twenty-five ml. of the chloroform phase was collected in a 50 ml. beaker and reduced to near dryness under a gentle stream of nitrogen in a steam bath. The residue was quantitatively transferred by 3 chloroform washings to a 4 dram, foil lined screw cap vial and reduced to dryness

⁷S and S, #588, Arthur H. Thomas Company, Philadelphia, Pennsylvania.

⁸Whatman No. 1, 18.5 cm. supplied by Fischer Scientific Company, Pittsburgh, Pa.

⁹Isolator/Lab., Fischer Scientific Co., Pittsburgh, Pennsylvania.

¹⁰Modified Eimer and Amend, Fischer Scientific Co., Pittsburgh, Pa.

again under nitrogen. The vials were tightly closed and stored at 0 C until quantitative assays could be performed on TLC plates.

Preparation of TLC plates. Three; 20 by 20 cm. glass plates were placed on a flat surface, wiped off with chloroform and secured by application of Scotch filament tape along the edges of two sides. Twenty Gm. of MN Silica Gel G-IR¹¹ in 40 ml. distilled water was vigorously shaken for 30 seconds in a glass stoppered flask, immediately poured on the plates and spread evenly with a 10" by 1 cm. glass rod. The bordering tape prevented contact between the plate and rod resulting in, approximately, a 250 u thickness of silica gel. After air drying for 10 minutes the tape was removed and the plates were placed in a storage rack¹² for 4 to 5 days prior to use. In humid weather the plates were placed in a 250 F oven for 15 minutes to facilitate moisture removal just prior to use.

Thin layer chromatography. Five ml. of chloroform was accurately pipetted to the vials containing the extract material. With a 10 microliter syringe¹³ and a spotting template, 1.0, 3.5, two 5.0 and 6.5 microliter portions were spotted on the plate 4 cm. from the bottom. On the same plate 3.5, 5.0, 6.5 and 10 ul. of quantitative and 5 ul. qualitative standards were also spotted. Five ul. of the standards were spotted directly over one of the 5 ul. sample spots to serve as an internal standard.

¹¹Brinkman Instruments, Westbury, New York.

¹²Warner-Chilcott Model 200-3, Scientific Products, North Kansas City, Mo.

¹³Model 701, Hamilton Company Inc., Whittier, California.

Plates were developed in an unlined unequilibrated glass tank¹⁴ equipped with a trough containing approximately 50 ml. 1/9 acetone and chloroform, V/V, as the developing solvent. The tank was sealed and the plate was developed at 23-25 C for 40 minutes or until the solvent front reached 12 to 14 cm. beyond the application points. After the solvent was evaporated the plates were illuminated from below by placing the plate flat, coated side up on a long wave ultraviolet light¹⁵ in a darkened room. Protective goggles¹⁶ were worn while viewing the plates.

Aflatoxin B₁ quantitation was made by visually comparing the intensity of fluorescence of the unknown to that of the standard, using the formula, micrograms/liter = $(S \times Y \times V)/(X \times W)$, where S = ul. aflatoxin B₁ standard equal to unknown; Y = concentration of B₁ standard in ug./ml.; V = volume in ul. of final dilution of sample extract; X = ul. of sample extract spotted giving fluorescent intensity equal to S, the B₁ standard; W = ml. of sample used. G₁ concentrations were calculated in like manner. All assays were triplicated.

The quantitative standard, SA 3-4,¹⁷ was prepared from pure crystalline B₁ and G₁ which was dissolved in a chloroform solution and contained 3.0 ug./ml. of B₁ and 2.0 ug./ml. of G₁. When diluted for

¹⁴Model K4097, Kensington Scientific Corporation, Oakland, California.

¹⁵Blak-Ray model XX15, Ultraviolet Products Inc., San Gabriel, California.

¹⁶Blak-Ray ultraviolet contrast control goggle, *ibid.*

¹⁷Supplied by Dr. Leo A. Coldblatt, Southern Utilization Research and Development Division, A.R.S., USDA, New Orleans, Louisiana.

analytical purposes, the standard contained B_1 - 0.00057 ug./ul. and aflatoxin G_1 - 0.00035 ug./ul.

The qualitative standard, SA 5-1,¹⁸ was used primarily for establishing the resolution of individual aflatoxins and for facilitating their identification on TLC analysis. The diluted qualitative standard contained approximately, B_1 - 0.001, B_2 - 0.00025, G_1 - 0.001 and G_2 - 0.00025 micrograms per microliter.

The diluted standards were contained in sealed 4 dram vials which were placed in tightly covered jars containing several ml. of chloroform and stored in the freezer at 0 C. Standards were allowed to equilibrate in the unopened jars at room temperature prior to TLC spotting.

EXPERIMENTAL ANIMALS. In the first experiment, 37 two week old White Rock chicks¹⁹ were obtained from the Poultry Science Department, Kansas State University. There were approximately equal numbers of male and females which were identified by numbered wing tags. When the chicks were 17 days old they were placed at random into 7 groups.

Beginning May 1, 1967, groups A, B and C containing 7 birds each, received respectively, 3, 2 and 1 ml. of the toxic media per bird per day. Three birds each, in Groups D, E, and F received corresponding doses of control medium. The remaining 7 birds served as untreated controls.

The testing period was 6 weeks. After the first 2 weeks the dosage was increased by 1 ml. for all groups. Beginning the last 2

¹⁸Supplied by Dr. Leo A. Goldblatt, Southern Utilization Research and Development Division, A.R.S., USDA, New Orleans, Louisiana.

¹⁹Hatched by Cobb Inc., Siloam Springs, Arkansas.

weeks, the Group A dose was increased by 2 ml., Group B, 1.5 ml. and C, 1.0 ml. Control doses were increased at the same rate.

For the second experiment, 44 chicks of the same breed and strain were obtained directly from the breeder on the first day of hatch. At 14 days of age (June 7, 1967) these birds were also placed into 7 groups. Groups A, B, and C consisted of 8 birds each which received 1.5, 1.0 and 0.5 ml. of toxic medium daily. Groups D, E, and F contained 4 birds each and received corresponding doses of control medium. The remaining 8 birds served as untreated controls. In this experiment the dose was doubled every 2 weeks and again ran for 6 weeks.

All birds were fed the same ration²⁰ from hatching until death. The ration was considered to be well-balanced and contained no antibiotics. The only medicinal additive was a coccidiostat²¹ incorporated at the rate of 115 Gm. per 500 pounds of feed. Feed and fresh water were available to the birds at all times.

Birds were housed in a battery arrangement but were not segregated by groups. In each experiment, protected heating coils were placed in the batteries until the birds were 4 weeks of age. No vaccinations were administered.

Oral administration of the toxic and non-toxic media directly into the crop was accomplished with separate 5 or 10 ml. glass syringes equipped with a 4-inch polyethylene tube forced over the shaft of a 1-inch, 12 gauge needle.

Weights of the birds were recorded on the first day of each experiment and every 7 days thereafter or at death.

²⁰Broiler Ration, code P-20, Department of Grain Science and Industry, Kansas State University.

²¹Amprol, Merck and Company, Rahway, New Jersey.

Birds in the first experiment were sacrificed by incising the jugular vein and were New York dressed in the poultry processing laboratory, Kansas State University. Birds surviving the second experiment were killed by manually severing the cervical spinal cord. All birds were necropsied at death or slaughter. Gross lesions were recorded and tissues were retained for histopathologic confirmation of aflatoxicosis. Sections consisting of a small portion of the right posterior lobe of the liver, lung, kidney, spleen and pancreas were fixed in 10% formalin and stained by hematoxylin and eosin.

The livers were wrapped in plastic bags, and placed with the legs and breast meat of the corresponding chickens and identified by wing numbers. Samples were held in a freezer at -15 C until extractions could be performed.

TISSUE ASSAYS.

Extraction of tissues. After thawing for 24 to 36 hours at 4 C, a total of 50 Gm. of muscle from the breasts and legs was finely diced, added to approximately 100 ml. of chloroform and emulsified in a blender²² for 4 minutes.

Except for the small portion retained for histopathologic examination, entire livers were weighed and treated similarly. The emulsions, liver and muscle, were transferred to 500 ml. glass stoppered flasks and the volume of chloroform was brought to 150 ml. and 250 ml.

²²Model 700-B Waring Blender, Waring Products Corporation, Winsted, Connecticut.

respectively. After sealing, the flasks were mechanically shaken for one hour, stored overnight in the refrigerator and assayed the following day using the method described by Eppley (1966).

Column Chromatography. A cleanup process was required to remove lipids and other extraneous fluorescing material prior to thin layer chromatography. Samples to be assayed were filtered through 50 cm. fluted filter paper. Fifty ml. of muscle tissue filtrate was collected for column chromatography while entire liver filtrates were used.

A ball of glass wool was placed loosely in the bottom of a glass column.²³ Chloroform was added until the column was approximately 2/3 full and the trapped air was removed by tapping the stopcock area. Five Gm. of granular anhydrous sodium sulfate was poured over the glass wool to serve as a base for 10 Gm. silica gel²⁴ which was dispersed in the chloroform by stirring. Settling was aided by drawing off some of the chloroform leaving 2 to 3 inches above the silica gel. Fifteen grams of sodium sulfate was slowly added above the silica gel partition and the chloroform was drawn off to the top of this layer.

The sample extract was added to the column and eluted at a flow rate of 10 to 20 ml. per minute with 150 ml. hexane followed by 150 ml. anhydrous diethyl ether. Aflatoxin, if present was finally eluted with 150 ml. methanol chloroform, 3/97, V/V.

²³Model 274-019, 19x300 mm chromatography column with Labcrest stopcock, Fisher and Porter Co., Warminster, Pennsylvania.

²⁴50-200 microns, Brinkman Instruments, Westbury, New York.

The latter fraction was collected from the time it was added until the flow stopped.

After evaporating to near dryness on a steam bath under a gentle stream of nitrogen, the residue was quantitatively transferred to a 4 dram vial and handled in the same manner as described for the medium extracts.

Thin layer chromatography. Thin layer chromatography was accomplished in a similar manner described for plating the medium extracts except that .5 ml. chloroform was added to the vial and 3, 5, and 10 micro-liter portions were spotted and compared to the same volumes of the standards. Standards were also spotted over one of the 5 ul. sample spots to serve as internal standards.

Although qualitative assays were of primary concern, approximate quantitation was made in positive cases by using the formula: $\text{ug./kg.} = (\text{SxYxV})/(\text{XxW})$ as described previously. In these calculations the value for W is expressed as Gm. of sample passed through the column.

All reagents used were ACS grade. Final cleaning of glassware was accomplished by rinsing with chloroform followed by acetone.

RESULTS

AFATOXIN ASSAYS OF YES MEDIUM. The medium in which the toxic mold was grown was shown to contain all 4 aflatoxins; B₂ and G₂ were present in very small amounts and were not assayed quantitatively. No fluorescing spots or interfering compounds were evident on TLC from the chloroform extracts of the control medium (Fig. 1). Further

Fig. 1 Diagram of chromatoplate illustrating TLC assays of toxic and control medium.

14 cm.		solvent front									
		Toxic Medium					Standards				
											Control Medium
8 cm.		0	0	0	0	0	0	0	0	0	- 0 Blue B ₁
7 cm.		0	0	0	0	0	-	-	-	0	- 0 Blue B ₂
6 cm.		0	0	0	0	0	0	0	0	0	- 0 Green G ₁
5 cm.		0	0	0	0	0	-	-	-	0	- 0 Green G ₂
microliters 1,3.5,5,5*,6.5		0	0	0	0	0	0	0	0	0	0
							3.5,5,6.5,10.5**				

cleanup by column chromatography was, therefore, unnecessary in this procedure.

The combination of batches 1, 2, and 3 toxic medium was shown to contain 45.5 micrograms of B_1 and 40 micrograms of G_1 per ml.

Batches 6, 7, 8, 9 and 10 yielded 114 and 70 ug./ml. of B_1 and G_1 , respectively.

EFFECTS OF AFLATOXIN ON EXPERIMENTAL ANIMALS. In the first experiment, 2 birds in Group A and 2 in Group B died by the end of the first week. Pecking became a problem after 2 weeks and the birds were debeaked on day 16. Two birds in Group A, 2 in Group B and 1 in Group C plus 2 control birds died from the resulting hemorrhage. Another bird from Group B died 30 days from the start of the test. The remaining birds survived the 6 week period.

All birds employed in Experiment 2 were debeaked prior to testing. Four birds died during the test period. All were in Group A, the highest dose level. The first died after 4 doses, 2 more died during the 3rd week and one bird survived 40 days but during that period gained only 59 Gm. in body weight. Four control birds were killed to serve as comparisons to 3 of those that died.

Birds dying (except those that died from debeaking complications in Exp. 1) made very little weight gain, if any, and prior to death, were listless and had a ruffled appearance. No other symptoms were observed.

Weight gains. In both experiments, birds receiving toxic medium at the two highest levels made significantly lower weight gains. While birds on the lowest dose levels did not gain as much as controls, the

difference was not nearly as great as that between those on the highest dose and the controls (Table 1). In general, the birds in Experiment 2 made somewhat greater gains than those in Experiment 1, possibly because of the difference in debeaking procedures.

The amount of toxin administered during the first 2 weeks was evidently the most important. After that period the dose was much greater for birds in Experiment 2 yet weight gains were not appreciably different between the corresponding groups.

Gross pathology. No gross lesions were observed in the esophagus, trachea, crop, lungs, brachial plexus, air sacs or sciatic nerves of any animal in either experiment. Only 2 control birds showed any pathology: No. 14637, Group D, Exp. 2, suffered an epiphyseal fracture of the distal right tibia on day 32 and No. 5987, Group G, Exp. 1, had calculi in the right kidney.

The most obvious changes were in the liver. The color varied from slightly pale to a very pale yellow. Although the yellow was observed primarily in livers of birds which survived high doses, the same color was observed in some of those receiving low doses. Affected livers tended to be firm with a mottled appearance and the borders were somewhat rounded. No evidence of nodule formation was observed. Affected livers had numerous small reddish spots, 1-2 mm. in diameter, diffusely scattered over the surface.

The kidneys and spleens were usually pale in affected birds. Enlarged, edematous kidneys and inflammation of the intestinal serosa were inconstant findings. Petechiation was observed in the epicardium of the 2 birds which died after 4 days under test.

Table 1. Comparison of daily dose and average weights in all groups of test animals.

Group	Beginning weight in Gm.	dose weeks 1 & 2 ml. (ug.)*	weight end of week 2 in Gm.	dose weeks 3 & 4 ml. (ug.)*	weight end of week 4 in Gm.	dose weeks 5 & 6 ml. (ug.)*	weight end of week 6 in Gm.
Tests							
A	199.9	3.0 (256.5)	329.0	4.0 (342.0)	547.0	6.0 (513.0)	807.5
A'	179.0	1.5 (276.0)	355.8	3.0 (552.0)	567.0	6.0 (1102.0)	897.4
B	193.2	2.0 (171.0)	317.0	3.0 (256.5)	487.0	4.5 (384.8)	670.8
B'	174.7	1.0 (184.0)	393.8	2.0 (368.0)	627.2	4.0 (736.0)	927.1
C	195.6	1.0 (85.5)	449.1	2.0 (171.0)	825.4	3.0 (256.5)	1208.5
C'	178.9	0.5 (92.0)	432.9	1.0 (184.0)	829.6	2.0 (368.0)	1252.2
Controls**							
D	197.2	3.0 -	470.0	4.0 -	896.0	6.0 -	1262.2
D'	178.9	1.5 -	506.1	3.0 -	995.3	6.0 -	1513.5
E	183.0	2.0 -	508.3	3.0 -	938.0	4.5 -	1293.8
E'	172.6	1.0 -	506.5	2.0 -	973.1	4.0 -	1500.5
F	195.8	1.0 -	553.5	2.0 -	990.2	3.0 -	1369.3
F'	162.5	0.5 -	492.6	1.0 -	967.7	2.0 -	1423.4
G	167.5	-	492.2	-	999.7	-	1408.8
G'	168.6	-	516.1	-	953.9	-	1397.0

A - G = Experiment 1 using Batches 1, 2 and 3 toxic medium in test animals.

A' - G' = Experiment 2 using Batches 6, 7, 8, 9 and 10 toxic medium in test animals.

* Total B₁ and G₁ in micrograms.

** Controls received non-toxic medium.

No lesions were observed in the skeletal muscles except for emaciation in acute cases.

Histopathology. Liver lesions were observed in all test animals. In those birds that died during the first week, the liver was characterized by severe hepatocytic degeneration, wide spread focal hemorrhages, congestion, slight fatty change and minimal bile duct proliferation.

In birds that died during the 3rd and 4th weeks there was extensive bile duct proliferation, severe central as well as peripheral necrosis, greatly increased fatty change, some hemorrhaging and evidence of regenerating hepatocytes in the form of circumscribed zones throughout the liver. The regenerating cells were arranged in a tubular fashion. Mitotic figures were commonly observed. Infiltration by lymphocytes and polymorphonuclear cells was also observed throughout the lobules.

After 5 weeks, livers of birds on the highest aflatoxin level were observed to have little or no evidence of bile duct proliferation and very little fatty change. There appeared to be an increase in hepatocytic regeneration. Focal areas of lymphocytic and mononuclear infiltration were increased particularly in the portal areas. A number of these aggregations contained many eosinophils.

Severity of lesions lessened with lower dosages and fatty vacuolation persisted for longer periods in livers.

In acute cases edema was evident in kidneys, spleen and pancreas. Tubular degeneration of the kidney appeared to be related to the degree of liver damage. The most consistent splenic lesion appeared to be the loss of definition of the lymphatic nodules and again was related to amounts of toxins received and was associated with the severity of

hepatic lesions. Necrotic foci in the pancreas were present in a small number of birds receiving the highest level of aflatoxin but no significance was attributed to this finding. No significant lesions were present in the lungs.

AFLATOXIN ASSAYS OF MUSCLE AND LIVERS. Of 45 birds receiving crude aflatoxins, 15 were shown to have aflatoxins or their metabolites in some form in muscle tissue and/or livers. The appearance of these toxins on chromatoplates is illustrated in Fig. 2. A summary of the data recorded concerning these birds is listed in Table 2.

All of these animals except the 3 in Group B, Exp. 2, died before the end of the testing periods. Only 1 bird reached what may be considered market weight.

Results of the TLC analyses are shown in Table 3. In most of the tissue extracts of control and test birds, a red fluorescing spot was observed on TLC at a point approximately 3 cm. beyond the point of application. Occasionally a yellow fluorescing spot was seen at a point about 2 cm. beyond the application point. Another white fluorescing material was observed to travel immediately below the solvent front.

DISCUSSION

Aflatoxicosis in varying degrees of severity was produced in chickens by daily administration of crude aflatoxins. Yields of aflatoxin used in these experiments are considerably less than those

Fig. 2 Diagram of Chromatoplate illustrating fluorescing spots of muscle and liver extracts and aflatoxin standards

14 cm. solvent front	Tests			Controls		
	Muscle	Liver	Standards	Muscle	Liver	
8 cm.	0 0	0 0	0 0	- 0	- 0	Blue E ₁
7 cm.	- 0	- 0	- 0	- 0	- 0	Blue E ₂
6 cm.	0 0	0 0	0 0	- 0	- 0	Green G ₁
5 cm.	- 0	- 0	- 0	- 0	- 0	Green G ₂
3 cm.	0 0	0 0		0 0	0 0	red
1 cm.	0 0	0 0		- -	- -	blue
.5 cm.	0 0	0 0		- -	- -	green
	0 0	0 0	0 0	0 0	0 0	application
	a.*b.**	c.*d.**	e.*f.*g.*h.**	i.*j.**	k.*l.**	points

* sample extracts; ** sample extracts plus standards; *** quantitative standard; *** qualitative standard. - no spots observed.

Table 2. Summary of data from animals found to be positive for aflatoxins on TLC.

Chick No.	Sex	Day 1 Weight Gm.	Weight at death Gm.	Days on experiment	Total mg. B ₁ & G ₁ received
Exp. 1					
<u>Group A</u>					
5628	M	199.5	214.0	7	1.80
5996	F	187.0	168.5	3	0.77
<u>Group B</u>					
5981	M	151.5	479.5	30	7.74
5978	F	191.0	406.0	15	2.66
5100	M	199.0	392.5	15	2.66
5983	M	222.0	241.5	8	1.37
5986	F	214.0	203.0	7	1.20
<u>Group C</u>					
5995	M	203.8	412.0	15	1.37
Exp. 2					
<u>Group A</u>					
14602	M	184.0	243.0	40	24.82
14605	F	119.0	102.0	3	0.83
14607	M	179.5	217.0	23	8.81
14608	M	211.0	453.5	23	8.81
<u>Group B</u>					
14609	M	168.5	1132.8	42	18.30
14610	M	192.0	942.0	42	18.30
14612	F	119.0	486.0	42	18.30

Table 3. TLC analysis of Positive animals

Chick No.	Muscle		G ₁	B.M. ¹	G.M. ²	Liver		
	(B ₁)	G ₁				(G ₁)	B ₁	B.M. ¹ G.M. ²
5620	--	--	--	--	--	--	--	+++
5996	7 ppb	4	--	--	--	17 ppb	10 ppb	++
5981	--	--	--	--	--	--	--	++
5978	2 ppb	2	--	--	--	16	11	--
5100	--	--	--	--	--	--	--	++
5988	16 ppb	15	--	--	--	21	20 ppb	++
5936	--	--	--	--	--	--	--	++
5995	--	--	--	--	--	--	--	+
14602	285 ppb	175 ppb	++	++	++	81 ppb	50 ppb	++++
14605	11 ppb	73 ppb	+++	+++	+++	57 ppb	55 ppb	+++
14607	57 ppb	55 ppb	++	++	++	30 ppb	4 ppb	++
14608	3 ppb	5 ppb	--	--	--	12 ppb	7 ppb	++++
14609	--	--	--	--	--	--	--	+
14610	--	--	--	--	--	--	--	--
14612	--	--	--	--	--	--	--	+

1Blue metabolite

2Green metabolite

+ Faintly fluorescent

++ Distinctly fluorescent

+++ Strongly fluorescent

++++ Very strongly fluorescent

- No fluorescence

reported by Davis et al. (1966a). Yields were greater in batches 6-10 because washing of mats and flasks with distilled water was not performed in the filtration processes as it was with batches 1-3. Evidence of the disease was confirmed by diminished weight gains, gross and microscopic lesions, and recovery of a fraction of the toxins by extraction and chromatography.

No distinct correlation could be made as to gross liver appearance and dose level. The liver lesions observed in these experiments closely resemble those described by Carnaghan et al. (1966). However, it is suggested that the petechiation they described may have been, in fact, islets of regenerating parenchymal cells.

It was shown in this study that aflatoxins and their metabolites can be demonstrated in skeletal muscle and livers of affected chickens. These findings, however small, are in contrast to those reported by Platonow (1965) who was unable to demonstrate the toxins or their metabolites in muscle and livers of White Leghorn chickens fed aflatoxin contaminated peanut meal.

No quantitation could be made of the metabolites demonstrated since standards for their comparison were not available.

It is safe to assume, however, that the blue and green spots which were observed on TLC plates from the birds receiving aflatoxin only are metabolites of B₁ and G₁. These metabolites are, in all probability, identical to those described by Nabney et al. (1967) designated as M₁ and M₂. Final proof of the toxicity of the metabolites demonstrated in these studies would have to be determined by bioassay.

In most cases, recoverable aflatoxins or their metabolites were confined to birds that died from the effects of aflatoxin or were from those which made very small weight gains. Only one bird of these 15 was considered to have reached market weight, and in view of the small amount of metabolite demonstrated and considering the small amount of toxins recovered in the other birds, this finding may not have been significant.

Aflatoxin in amounts less than 50 ppb. are considered insignificant.²⁵ Tissues in only 3 of the test birds were shown to contain the toxins in excessive amounts. None of these birds weighed more than 250 Gm. and all were on the highest level.

Toxins were recovered in 3 of the 5 test birds who died after debeaking. It is difficult to say what the results would have been had these birds survived. It is likely that no toxins would have been demonstrated because at the time of their death they were making weight gains comparable to those in the same groups who did survive and in whose tissues no toxins were demonstrated.

It would seem, therefore, that birds reaching market weights and undergoing inspection would present no health hazard even though they had ingested considerable amounts of aflatoxin.

On the other hand, it may be that the toxins cannot be quantitatively extracted from animal tissues as they can be from other foods such as peanuts and peanut products. Certainly this possibility warrants further investigation. Careful excretion studies would have

²⁵Mr. Humberto Guerrero, Chemist, FIA Regional Laboratory, Dallas, Texas 75204. Personal communication, March, 1957.

to be made in the avian species to measure the efficiency of these procedures as applied to tissue extraction.

Furthermore, the author is of the opinion that the cleanup process should be modified so as to eliminate the other fluorescing materials found on TLC in this study.

CONCLUSIONS

Chicks appear to be most susceptible to aflatoxins during the first 4 weeks of life.

Aflatoxins and/or their metabolites can be extracted from livers and skeletal muscle of White Rock chickens suffering from aflatoxicosis.

The consumption of flesh from animals who have ingested aflatoxins cannot be disregarded as public health hazard until the toxic effect of these compounds has been established in the human and the metabolism and excretion of the toxins in animals is understood.

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AFLATOXICOSIS: THE PRESENCE OF AFLATOXINS OR THEIR
METABOLITES IN LIVERS AND SKELETAL MUSCLE OF CHICKENS

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Little information has been reported in the literature regarding the fate of aflatoxins in animals ingesting the toxin in food of animal origin. However, a toxic substance has been reported in milk of animals fed aflatoxin contaminated rations.

In this study the possibility of the presence of aflatoxins or their metabolites in livers and skeletal muscle of chickens was investigated in the interests of public health.

Aspergillus flavus ATCC 15517 was maintained and used for aflatoxin production in liquid YES media according to the methods described by Davis, Diener and Eldridge (1966). After filtering off the mycelial growth, the filtrate was assayed for aflatoxins B₁ and G₁ and administered orally to two week old White Rock chicks in varying doses daily for 6 weeks. Liquid YES medium was also given in corresponding doses to control birds while other birds received no media. All birds were maintained in a battery, but test and control birds were not separated. Live weights were recorded at the beginning of the tests and at 7 day intervals thereafter. Weight gains decreased notably as doses of the toxin increased. All birds were necropsied at death or slaughter. Sections of liver, kidney, spleen, pancreas and lungs were prepared for histologic examination. Breast and leg musculature was saved separately from the livers and samples were frozen at -15 C until assays could be performed. After thawing 24-36 hours at 4 C, 50 Gm. muscle were finely diced, placed in a suitable amount of chloroform and mixed in a blender. Entire livers were treated similarly. The emulsions were shaken for 1 hour, held overnight in the refrigerator and assayed for aflatoxins using the method described by Eppley (1966).

Of the 45 birds receiving aflatoxin, 15 were found to have aflatoxins or their metabolites in livers and/or the muscle. Most of these birds died of the disease or made slight weight gains. Only one of these birds reached market weight and it was shown to contain a small amount of metabolite in the liver.

The results of these studies tend to indicate that aflatoxins or their metabolites in muscle and livers of affected animals are present in insignificant amounts. Further work is warranted to determine the quantitative amounts actually present in tissues.